[redacted]

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Date
December 22, 2015

From [redacted]

To [redacted]

cc [redacted]

Survival study of *Saccharomyces cerevisiae* GE strain versus wild type in surface water and soil [redacted]

1. Summary

A survival study has been performed with 3 different *Saccharomyces cerevisiae* strains in soil and surface water.
The wild type parental strain [redacted] has been compared with the GE strain [redacted] and with the non-GMO strain [redacted], well used in the ethanol industry and insourced from Fermentis/Le Saffre. This strain is named BIE124. Both sterilized and non-sterilized soil and surface water samples from Delft has been tested at two temperatures: 8°C and 25°C.
The final results of the experiment indicates that the genetically engineered strain GE [redacted] showed no significant advantage in survival or outgrowth compared to the indigenous flora under any circumstances tested. There is also no development in outgrowth observed of all the yeast strains present in the non-sterile soil samples stored at 8 and 25°C. There is a strong decrease visible of all the yeast strains compared to the indigenous flora examined in the non-sterile water samples. The presence of the yeast strains in the non-sterile soil samples remains stable after inoculation and compared to the indigenous flora no outgrowth is observed.

Contents

2. Introduction

A newly developed GE-strain has acquired the ability to ferment [redacted]. Before approval for use of this new strain can be given, prove needs to be generated that this new strain has no intrinsic advantage over wild type yeast to outgrow and flourish in the environment if containment is breeched. Earlier research with comparable strains showed that the GE-strains did not contain a survival advantage in the environmental samples over the wild type *Saccharomyces cerevisiae* yeast and indigenous microorganisms.

3. Experiment

3.1. Environmental samples

3.1.1. Surface water

Surface water samples were collected directly from the "Delftse Vliet" in Delft, The Netherlands. Half of the surface water samples were sterilized for 20 minutes at 121°C (effectively).

3.1.2. Soil

Soil samples were collected from the DSM site in Delft. The soil was dried at 25°C for one week before sieving (1mm). The soil was divided in two and one half was sterilized for 5 hours at 160°C (effectively).

3.2. Microbial preparations

- [redacted]

3.3. Yeast inoculum

Frozen vials from SCU were used for preparation of the inoculum
see procedure below:

- inoculate 40ml PCB with 100 microliter from the SCU vial
- incubate in a shake incubator at 32°C, 250 rpm
- Determine a rough cell count under the microscope.
- Inoculums are ready for further use.

3.4. Inoculation of the samples

The environmental samples (surface water, soil; sterilized and non-sterilized) were inoculated with the mentioned yeast strains in triplicate. The inoculations were be done using a single strain, as mixing the strains will make it very difficult to distinguish them, effectively making it impractical to separate them again to see differences in survival rate. As a control, samples without inoculation were also made in order to compare with indigenous flora.

The level of inoculation was [redacted] The exact number of viable cells in the samples were measured during the first t=0 analysis. The samples were stored at two temperatures; 8°C and 25°C.

3.5. Microbiological analyses

Throughout the experiment, all samples were cultivated using Oxytetracycline Glucose Yeast extract agar (OGY) with Oxytetracycline to selectively detect the present yeast cells. The plates were incubated at 25°C for ~5 days before counting and recalculation to cfu/ml or gram.

The indigenous population of microorganisms present in the non-sterile samples was done on PCA with and without natamycin (to inhibit growth of the yeast inoculation strains). These plates were incubated for 3 days at 30°C. After incubation, the number of viable cells (formed colonies) were counted and recalculated to cfu/ml or gram using the chosen dilution.

The enumeration method was done by making decimal dilution in stylized and buffered Physiological Salt solution (0.89% NaCL) before testing 1ml dilution using direct pour-plates.

3.6. Analytical time points

The samples were analyzed at the time-points summarized in Table 1. Table 2 shows the conditions used to test the GMO and control strains.

Table 1: Analytical time-points

T	Timing
t=0	0 days
t=1	1 week
t=2	2 weeks
t=3	3 weeks
t=4	4 weeks

Table 2: Conditions used for the experiments.

Water Sterile 8°C WT	Water Sterile 8°C GMO	Water Not Sterile 8°C WT	Sterile 8°C GMO	Soil Sterile 8°C WT	Soil Sterile 8°C GMO	Soil Not Sterile 8°C WT	Soil not Sterile 8°C GMO
Water Sterile 25°C WT	Water Sterile 25°C GMO	Water Not Sterile 25°C WT	Sterile 25°C GMO	Soil Sterile 25°C WT	Soil Sterile 25°C GMO	Soil Not Sterile 25°C WT	Soil not Sterile 25°C GMO

The sterile samples were examined at the presence of yeast (GEO or WT) in case there is an yeast inhibiter present in the water or soil.

4. Results

4.1. Graphical presentation

In Figure 1 through Figure 4 the average of the measured cfu's per time point (WT, GEO & indigenous flora) is presented.

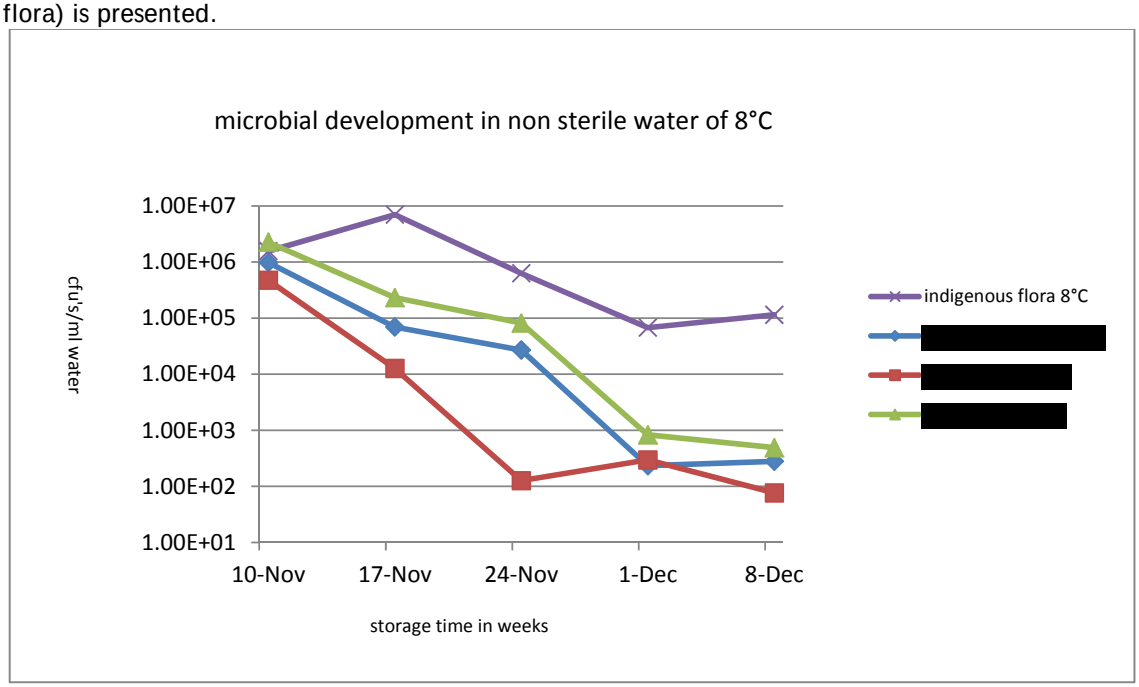

Figure 1: Microbial development in Non-Sterile water of 8°C.

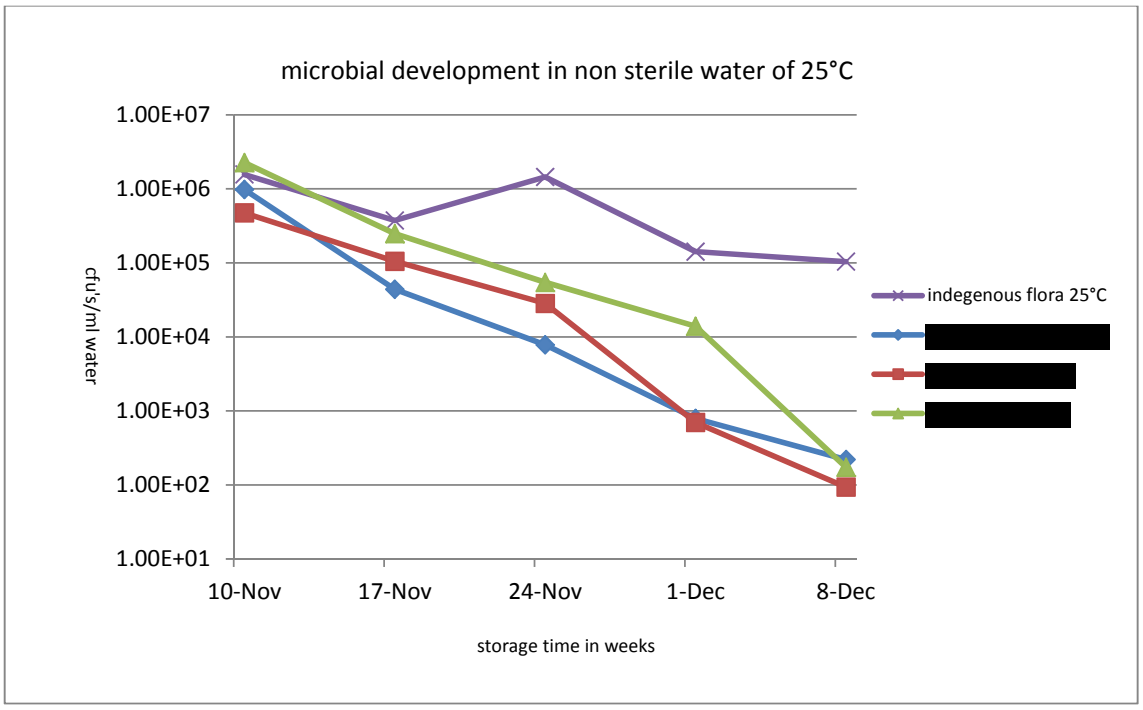

Figure 2: Microbial development in Non-Sterile water of 25°C.

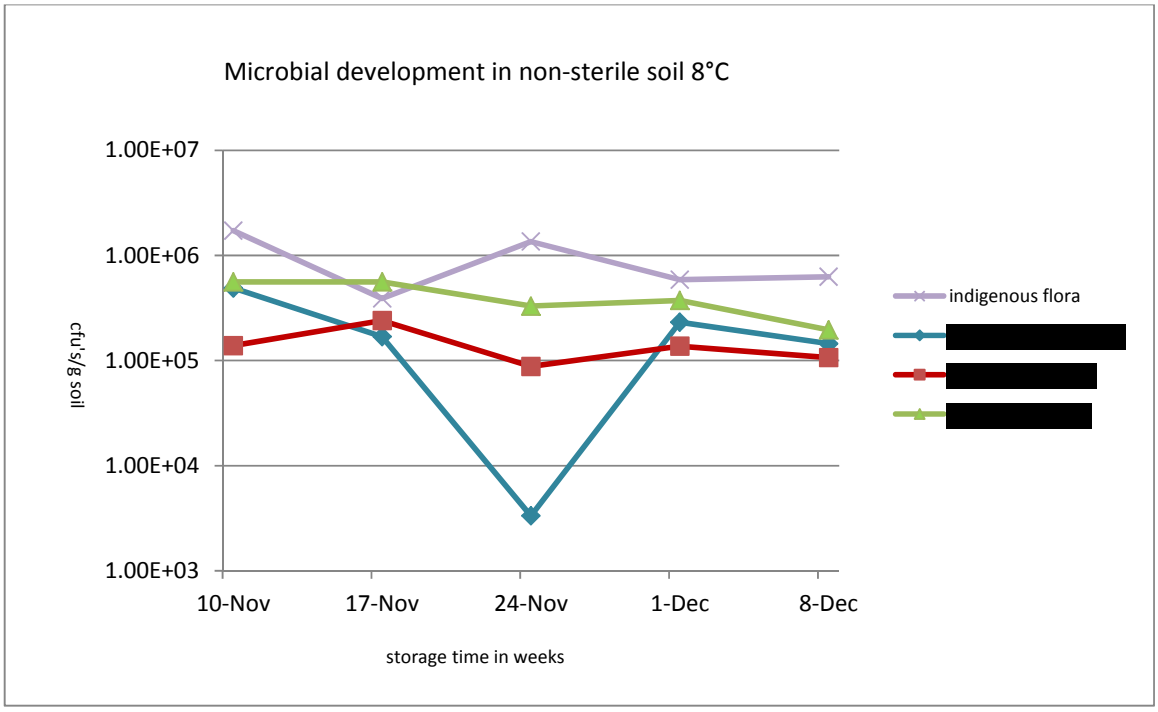

Figure 3: Microbial development in Non-Sterile soil of 8°C.

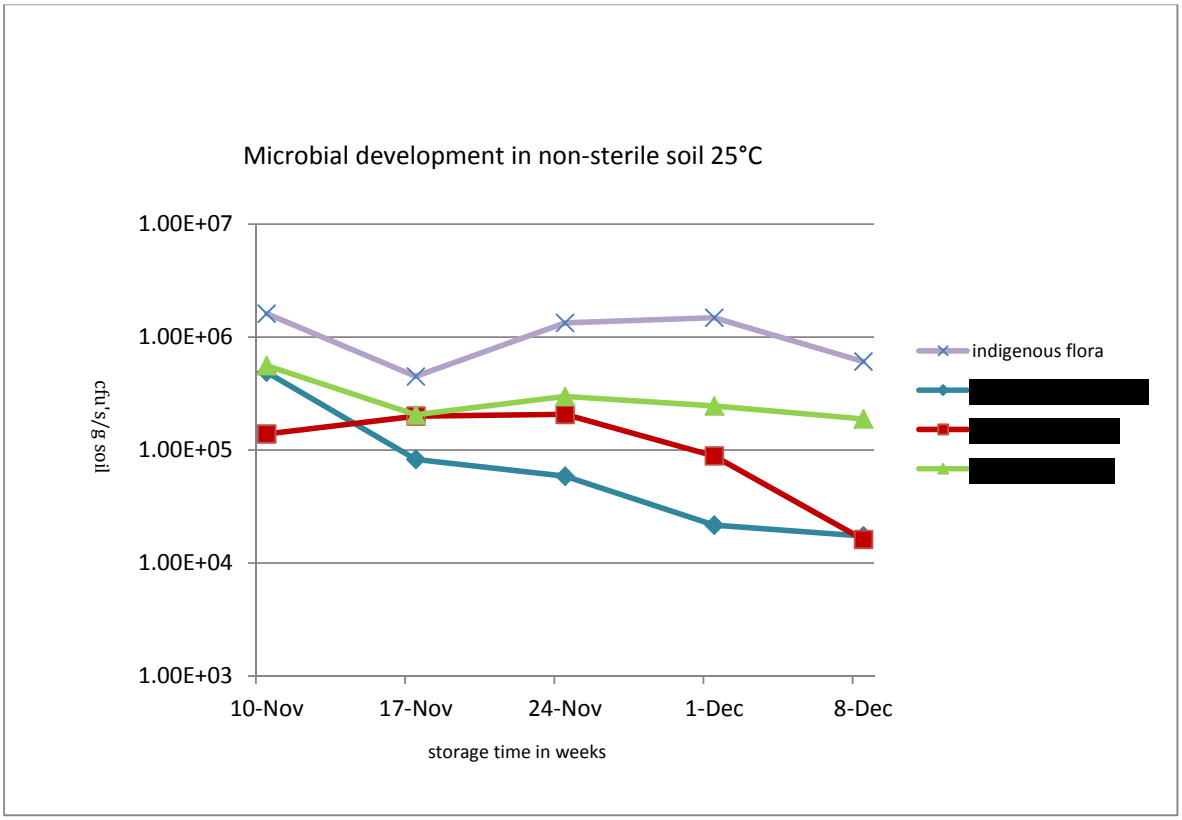

Figure 4: Microbial development in Non-Sterile soil of 25°C.

4.2. Statistical assessment

A full statistical assessment has been performed and reported in the memo embedded in the Appendix. Calculations show that a difference of 0.44 on log10 scale can be detected with a 95% confidence interval. This means that the log10 count difference between 2 strains is 0.44 or larger, that it is statistical significant. In none of these cases, the absolute difference exceeded the biological relevant difference 1 on log10 scale. Therefore, there was no biological relevant outgrowth by the GMO as compared to the two reference strains.

5. Conclusion

Compared to the indigenous flora all the yeast strains ([redacted]) showed no outgrowth during the run time of the experiment. The results confirm that the [redacted] tested have no competitive advantage (relative to the indigenous flora) when accidently released into the environment.

6. Appendix

The following document contains the complete statistical analysis of the data.

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Memo

DSM Biotechnology Center

Alexander Fleminglaan 1
2613 AX Delft
Netherlands

Date
December 20, 2015

From [redacted]

To [redacted]

cc [redacted]

Advanced Yeast Environment testing

Summary
Strain [redacted] was tested against reference strains [redacted] under different environmental conditions to test the GMO growth ability. The results show that the GMO does not have a biological advantage as compared to both reference strains.

1. Introduction

Strain [redacted] was tested against reference strains [redacted] in an environmental study to determine its growth behavior under different circumstances. The experimental setup of this study has been described in [redacted]. This document reports the statistical outcome. The results in this document answer the following questions:

- Does [redacted] significantly outgrow reference strain [redacted] 4 weeks of growth?
- Does [redacted] significantly outgrow reference strain [redacted]?
- If there is a significant higher count for [redacted], does it exceed a difference of 1 on a log10 scale?

2. Data Analysis

2.1. Data preprocessing

The data were provided in tables as shown in Table 1. The tables were transformed into one 'long' table to allow for easy handling.

Table 1: example table for Water Sterile @ 8 degrees Celsius, Yeast (OGY) as background.

[redacted]	Yeast (OGY)				
Water Sterile 8C	Cell counts (cfu/g or ml)				
Date of analysis	10-Nov	17-Nov	24-Nov	1-Dec	8-Dec
Repetition | t=	0	1wk	2wk	3wk	4wk
1	5.60E+05	3.20E+05	3.20E+05	1.00E+05	1.32E+05
2	6.40E+05	3.52E+05	2.56E+05	1.20E+05	1.50E+06
3	4.80E+05	3.52E+05	2.40E+05	1.00E+05	1.40E+06
Averages	5.60E+05	3.41E+05	2.72E+05	1.07E+05	1.01E+06

In total, 8 counts were detected that did not fit with the expected count. Those counts were replaced by the average count of the two other measurements or (in case of a non-detect for two counts, were replaced by the count of the remaining measurement). The replacements are summarized in Table 2.

Table 2: Data records with issues and resolution to solve the apparent large residuals.

Strain	Experiment	Week	Temperature	Repeat	Issue	Resolution
[redacted]	Water Non-Sterile	2	25C	1	Unexpected low count	Replaced by average of other two measurements
[redacted]	Soil Sterile	0	8C	1	Unexpected low count	Replaced by value of repeat 3
[redacted]	Soil Sterile	0	8C	2	Unexpected low count	Replaced by value of repeat 3
[redacted]	Soil Sterile	1	8C	1	Unexpected low count	Replaced by value of repeat 3
[redacted]	Soil Sterile	1	8C	2	Unexpected low count	Replaced by value of repeat 3
[redacted]	Soil Sterile	3	8C	3	Unexpected low count	Replaced by average of other two measurements
[redacted]	Soil Sterile	0	25C	1	Unexpected low count	Replaced by value of repeat 3
[redacted]	Soil Sterile	0	25C	2	Unexpected low count	Replaced by value of repeat 3

2.2. Assessment of variation and confidence limit

In order to determine possible differences between cell counts, the variability of the method needs to be determined. A visual check was performed whether the log10 count residual error is dependent on the magnitude of the log10 count. The result of this is shown in Figure 1 and indicates that there is no dependency. As a result, we can determine an overall standard deviation for the statistical assessment. This standard deviation (in terms of log10 counts) is determined to be 0.19(5133). This standard deviation is close to the value found in an earlier study (0.17, [redacted]). We aim to test whether there is a significant increase (95% on-sided confidence limit) in log10 count in the following comparisons:

- [redacted]

Figure 1: Absolute residual log10 count versus their average log10 count. The residuals are determined by subtracting the average of three measurements.

In each comparison, the [redacted] dataset is used twice and therefore we need to correct for multiple testing. The classical approach 'Bonferroni' is chosen in which the critical p-value is divided by the number of tests. So, we are going to test with this confidence limit which has a z-value of 1.96 for one sided tests. A difference of 0.44(1627) or more on a log10 scale would then be a significant difference. This value is determined according to Equation 1. In this equation, the standard deviation is divided by the square root of three because of the replicate measurements resulting in a more precisely determined average value for the count. The value 2 is included to take into account that both estimated log10 count values have their individual confidence limit. Only when they overlap, then the determined difference is larger than the critical difference.

$$Critical\ difference = 1.96 * 2 * \left(\frac{0.19533}{sqrt(3)}\right) = 0.441627 \quad (1)$$

3. Results

Table 3 and Table 4 show how [redacted] compare to the two reference strains [redacted] In case of a log10 count for the GMO which is significantly larger than the determined critical value, the cell is highlighted. For each significant difference a more detailed comparison is performed to determine whether the shown situation is present during all weeks.

Table 3: Statistical results for comparison between [redacted]. Red shaded areas indicate that a significant higher log10 count was found for [redacted]

t=4 weeks		[redacted]		
Experiment	Temperature	All (PCA)	Indigenous bacteria (PCA +Nata)	Yeast (OGY)
Water Sterile	8			0.82
	25			0.04
Water Non-Sterile	8	-0.55	-0.11	-0.56
	25	0.6	0.64	-0.34
Soil Sterile	8			-0.13
	25			-0.24
Soil Non-Sterile	8	0.87	-0.06	0.22
	25	-0.29	-0.03	0.14

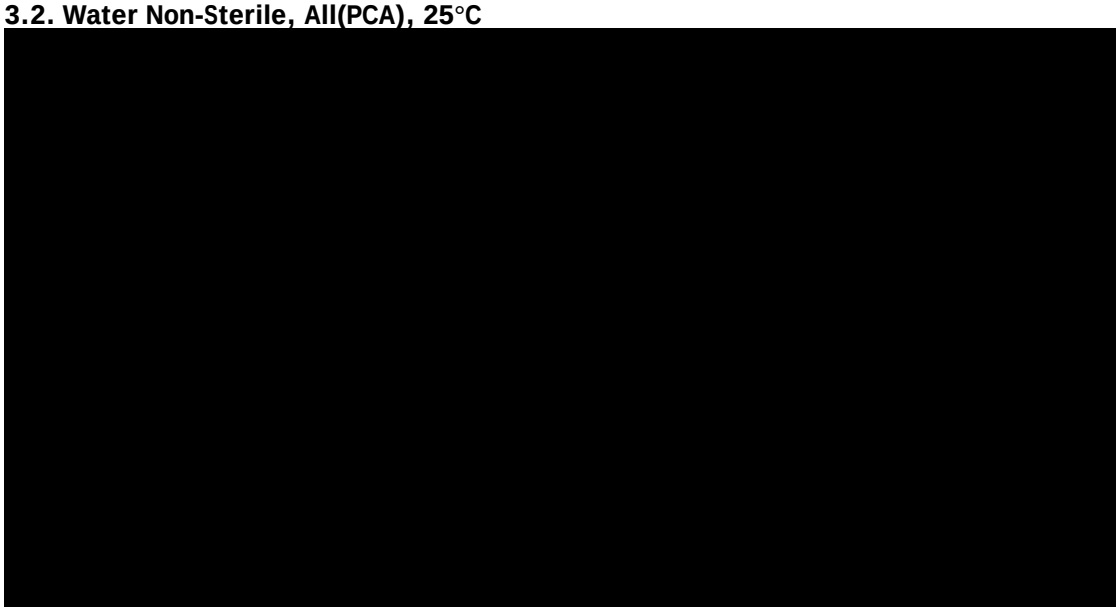
Table 4: Statistical results for comparison between [redacted] Red shaded areas indicate that a significant higher log10 count was found for [redacted]

t=4 weeks		BIE_124		
Experiment	Temperature	All (PCA)	Indigenous bacteria (PCA +Nata)	Yeast (OGY)
Water Sterile	8			0.2
	25			-0.71
Water Non-Sterile	8	-0.24	-0.08	-0.8
	25	0.21	0.58	-0.25
Soil Sterile	8			-0.22
	25			-1.16
Soil Non-Sterile	8	0.18	-0.2	0.01
	25	-0.11	0.41	0.04

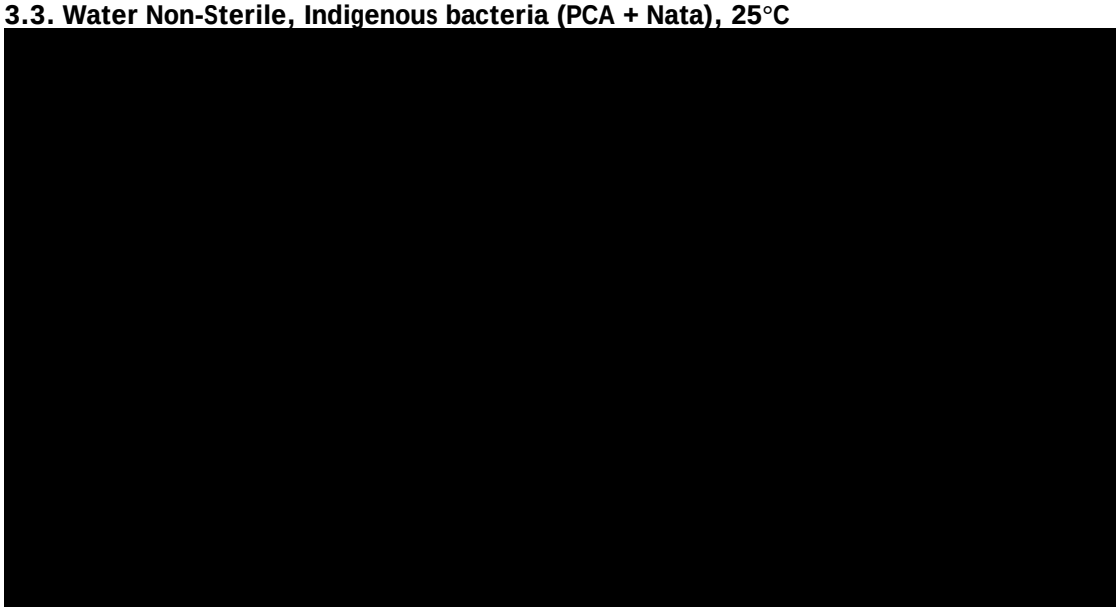
3.1. Water Sterile, Yeast (OGY), 8°C

The results show that for all cases, [redacted] does not have significant higher log10 counts than BIE_124 and that increasing or decreasing log10 counts correlate with the reference strains.

3.2. Water Non-Sterile, All(PCA), 25°C



3.3. Water Non-Sterile, Indigenous bacteria (PCA + Nata), 25°C



3.4. Soil Non-Sterile, All (PCA), 8°C

4. Conclusion

The following questions were researched:

- Does [redacted] have a statistical significant higher count than reference strain [redacted] after 4 weeks of growth?
    - Yes, as indicated in Table 3 in four cases
- Does [redacted] have a statistical significantly higher count than reference strain [redacted]
    - Yes, as indicated in Table 4 in one case
- If there is a biological significant higher count for GMO-YDO1545-CA, does it exceed a difference of 1 on a log10 scale?
    - No.